MUTATION OF BACTERIOPHAGE WITH RESPECT TO TYPE OF PLAQUE

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ONLY a few instances of well defined mutation have been described for the bacteriophages. The best known form a class of mutations affecting host range, recently studied by LURIA (1945a, 1945b), whose papers may be consulted also for mention of the earlier literature.

This paper is concerned with a second class of mutations, occurring within single plaques of certain coli-dysentery phages, where the appearance of the mutant type occasionally gives rise to variegated plaques. From the variegated plaques,the mutant is readily isolated by virtue of its distinctive plaque type and proves to differ from the parent type in causing prompt rather than delayed lysis in undiluted culture, but to be nearly identical with it otherwise in growth characteristics, host range, and antigenic specificity.

The new mutation has been described in a preliminary note (HERSHEY and BRONFENBRENNER 1945), and the existence of the two types of phage ("T4a" and "T4b") in lysates has been mentioned by DEMEREC and FANO (1945). A series of mutations affecting plaque type, which like ours was not related to adaptation to resistant bacterial strains, was noted also by SERTIC (1929). Its relation to the mutation described here is not clear from the published description.

NOTATION

The system of naming mutations of bacteriophages to be used in this paper departs somewhat from systems previously used. It has been developed during the past year by a group of interested workers (M. DEMEREC, M. DELBRÜCK, S. E. LURIA, A. H. DOERMANN, and the writer) in an attempt to combine convenience and descriptiveness within the usual genetic convention. It is to be hoped that it is a step toward a uniform nomenclature for the bacterial viruses.

Wild type phages are to be designated, following the notation of DEMEREC and FANO (1945), by numbers preceded by the letter *T* (for type)—for example, *T1*. The letter *T* may further be taken as a generic designation for phages acting on *Escherichia coli* strain *B* (and, so far, also on many strains of Shigella). The numbers are arbitrary, with the single restriction that the even numbers should be reserved for the antigenically and morphologically related phages (DELBRÜCK 1946a) already represented by *T2*, *T4*, and *T6*. These are homologous with the serological group II of BURNET (1933a), the "WLL" of SCHLESINGER (1934), and the "PC" of BRONFENBRENNER (KALMANSON and BRONFENBRENNER 1939). They evidently form a very commonly studied group among the coliphages. As will be seen presently, they also have functional and mutational properties in common.

At present the numbers *T1* through *T7* are permanently occupied (DEMEREC and FANO 1945). For the purposes of the present study, we are adding an additional wild type (the "AC" of BRONFENBRENNER'S collection), which we shall call *T16*.

The derivatives of *T2* to be mentioned in this paper will serve to illustrate the new system of naming mutants of the bacterial viruses.

It sometimes happens that a given type of phage gives rise spontaneously during propagation to a line differing from its progenitor. When this happens, one has the choice of throwing it away, giving it an arbitrary designation, or working out its mutational relationships. Since the first is not always desirable, and the last not always possible, it is necessary to have some system for designating phages which are neither authentic wild types (that is, phages certainly not nearly related to other known phages), nor mutations obtainable at will. For this type of variant, and where the study of host relationships and antigenic properties supports the inference as to generic relatedness,we use capital letters to indicate the subtypes. For instance, the three lines of putative PC phage established by KALMANSON, LURIA and HERSHEY prove to be genetically different, but certainly very closely related. These will be called *T2K*, *T2L*, and *T2H*, respectively, and will be described further below. For the present, owing to the nature of the material, these symbols are not likely to be confused with the notation for the dominant genetic factors of classical genetics.

For authentic, independent genetic factors, we shall use small letters descriptive of the observed mutation away from wild type. Two of these will be mentioned here.

One class of mutants are those characterized by altered host range, the prime mutants in the notation of LURIA (1945a). These have usually been isolated by means of specifically resistant variants of the sensitive bacterial strains. (The nomenclature for the bacterial mutants themselves is illustrated by the example *R/3,2,4,7*, which means a derivative of *R* selected for resistance to *T3*, proving to be resistant also to *T2*, *T4*, and *T7*, but to no other virus of the *T* system (DEMEREC and FANO 1945)). Mutant phages, differing from the parent type by their ability to infect bacteria resistant to it, will now be designated by the letter *h* (for the altered host range), followed when necessary by the symbol in parentheses for the bacterial strain which served for the isolation. Thus *T2H* plated on *R/2H* yields *T2Hh(R/2H)*. Among these there are two varieties: h^t (for turbid plaques) and h^c (for clear), which differ both in appearance of plaques on *R/2H*, and in infectivity for this host relative to that for the fully sensitive bacterium. It is not yet known whether h^c and h^t are separate loci.

The new mutation, corresponding to a second genetic factor, will be designated by the letter *r* (for rapid lysis)—that is, *T2Hr*. When *T2Hh* undergoes *r* mutation, we get *T2Hhr;* when *T2Hr* undergoes *h* mutation, we get *T2Hrh*. If it is desired to specify the wild type alleles of these characters, we use $T2Hr^+$, $T2Hh^+$ or $T2Hr^+h^+$. Usually the symbol *T2H* alone will identify wild type explicitly enough.

MATERIALS

Bacterial strains

Our stock of sensitive *E. coli* (the PC of BRONFENBRENNER) differs from the strain *B* derived from the same source by DELBRÜCK and LURIA (1942) by at least one mutation, since it is sensitive to a killer substance (cf. GRATIA 1925) produced by another strain of *E. coli* to which *B* is resistant. We therefore designate our stock by the letter *R* (for rough). We prefer *R* to *B* for mutational studies owing to the usefulness of the additional marker, which proves to be independent of virus sensitivity. Another derivative of the same culture, called *S* (for smooth), we prefer to *R* for propagation of phage stocks and for plaque counts, since *S* grows to higher populations in broth and provides a whiter background in seeded agar plates. A fourth derivative of the same culture, known as *H*, originally selected for its tendency to yield sterile lysates with *T2H*, proves to adsorb this phage better than does *R*, and is therefore used in some of the experiments cited below. It seems necessary to record these details, which serve at least to call attention to the difficulty of preserving uniform biological materials. In general, however, the *T* system of phages act identically on the bacterial strains mentioned, so that the casual reader may consider the terms *R*, *S*, *H*, *B*, and "sensitive bacterium" as synonymous.

New phages

The new strains of phage mentioned in this paper originated as follows:

T16. A line stemming from a single plaque of the "AC" phage of BRONFENBRENNER.

T2H. A line established by the writer, stemming from a single plaque of the "PC" phage of BRONFENBRENNER. This line has been described as "P9H" (HERSHEY, KALMANSON, and BRONFENBRENNER 1944).

T2K. A line established by KALMANSON, stemming from a single plaque of the same "PC" phage, otherwise called "PC" (KALMANSON and BRONFENBRENNER, 1939).

T2L. The phage gamma of DELBRÜCK and LURIA (1942) was established by them from a single plaque of *T2K* and will now be called *T2L* to distinguish it from the lines mentioned above. This strain is the original *T2* (DEMEREC and FANO 1945). We also have obtained a phage similar to or identical with *T2L* from *T2K*.

The three lines of *T2* breed true on sensitive bacterial strains. It is not known certainly that they have a recent common parent, though this is very probable in view of their origin and close similarity. At any rate they are not known to represent "wild types" of remotely independent origin, and the arbitrary notation suggested above serves to indicate this fact, until their mutational relationship has been worked out.

The differentiation of the even-numbered phages can be made by means of the following mutants (among others, to be described elsewhere) isolated by us from the sensitive bacterial strain: (*R/2H*); (*R/2H*, *2K*); (*R/2K*, *6*);

(*R/2H, 2L*); *R/2H, 2K, 2L*; *R/6, 16*; *R/3, 4, 7, 16*. The mutations indicated in parentheses are rare.

The close relationship between the *T2* lines is shown by the fact that the great majority of bacterial mutants resistant to any one are resistant to all, and by the inability of anti-*T2K* immune serum to distinguish them clearly.

The remoter relationship among the wild types *T2*, *T4*, *T6*, and *T16* is shown by the fact that all cross-react in antisera to any one, but with unmistakable quantitative differences (M. DELBRÜCK, personal communication, and unpublished experiments of the writer). Our classification of the even-

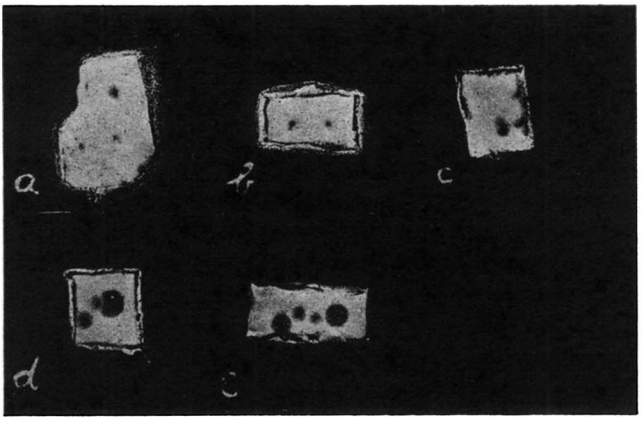

FIGURE 1.—Plaques illustrating *r* mutation. a. Plaques of *T2K*, wild type, and a mottled plaque arising from a bacterium infected with both wild type and *r* mutant. b. Two plaques of *T2K* wild type. The one to the left shows a typical sector of *r* mutant. c. Two contiguous plaques of *T2K* wild type. The lower one shows a very large sector of *r* mutant. d and e. Plaques from a mixture of pure stocks of *T2H* wild type and *r* mutant.

numbered phages is thus in accord with the data of BURNET (1933a), which show that wild type phages of a given serological group are nearly always neutralized more rapidly by homologous antiserum than by antiserum to related types.

ISOLATION OF THE *r* MUTANT

Plaques of *T2H* in very soft agar (that is, 0.6 percent "Difco standardized") in plates prepared by the agar layer method (HERSHEY, KALMANSON, and BRONFENBRENNER 1944) show a distinct halo of partial lysis visible at 18 to 24 hours. About one percent of the plaques show sectors of more or less complete lysis within the halo. Phage propagated from the aberrent sectors invariably yields, in addition to the parent type, *r* plaques of somewhat larger size, which differ in showing nearly complete lysis at the periphery. There is usually an intermediate ring of imperfect lysis in the *r* plaques. On further isolation, both types breed true except for further *r* mutations. In addition to the variegated plaques, platings of stocks of wild type may or may not show a few primary *r* plaques when practicable samples are plated. The proportion probably de-

pends on whether *r* mutations have occurred earlier or later during lysis.

Figure 1 shows photographs (made by exposing sensitized paper to light transmitted through slices of agar cut out and supported on cover slips) of plaques of wild type and *r* mutant. The contrast between the two types of plaque is especially well shown in the plating of a mixture of the two phages. The sharp boundary between overlapping plaques of the two kinds shows that the *r* mutant cannot lyse bacteria already inhibited by wild type.

Under slightly different conditions of plating, where the wild type plaques may lack the halo, the *r* plaques may show one (as found by DEMEREC and FANO 1945). In platings on the surface of one percent agar plates, neither type shows a halo, and there is only a difference in average size of plaques.

PARALLEL MUTATIONS

The *r* mutation occurs in an identical manner in all the phages so far examined which belong to the even numbered serological group—namely, the three lines of *T2* itself, several *h* mutants of these, and *T4*, *T6*, and *T16*. The only difference we have noted is that halo formation with wild type is more sensitive to conditions of plating with some phages than others. The halo is especially well marked with *T2H*. With this phage, and with *T4* and *T6*, the contrast between plaques of wild type and *r* mutant is very striking.

The even-numbered phages comprise a group of antigenically related small-plaque formers, whose morphology, as revealed by the electron microscope, is identical within the group (*T2L*, *T4*, and *T6*) (DELBRÜCK 1946a), but is easily distinguishable from that of unrelated phages. In addition, they have in common a peculiar lysis-inhibiting property, very noticeable in cultures made for production of phage stocks, where visible lysis ensues only some five or six hours after infection of all the bacteria with phage. This behavior stands in sharp contrast with the latent period of phage growth at high dilutions of bacteria and phage, which is only 21 to 26 minutes for several of these phages (DELBRÜCK 1946a, and unpublished experiments of the writer). According to experiments in progress of A. H. DOERMANN and M. DELBRÜCK (personal communication), it is likely that the delay is due to reinfection of the bacteria with phage liberated by the lysis of a few bacteria, which somehow interferes with the lysis of the remainder. The *r* mutants uniformly differ in causing complete lysis in about one hour under identical conditions. The other members of the *T* system (odd numbered from *T1* to *T7*, inclusive) do not cause lysis-inhibition and do not show a comparable mutation.

INDEPENDENT MUTATIONS

The occurrence of parallel mutations in several closely related strains of phage suggests at once that the same phage can undergo two or more mutations independently, for it is likely that the strains themselves differ from each other by one or more mutations. This supposition is confirmed by showing that the known *h* and *r* mutations of *T2H* correspond to independent genetic factors.

Mutants active on the bacterial strain *R/2H* (resistant to *T2H*) are isolated from *T2H* by plating about 5×10^8 particles of this phage in agar seeded with the resistant bacterium. Two types of mutant are obtained, both regularly appearing with about the same frequency in initially pure stocks of *T2H* and both breeding true on sensitive bacteria. These will be called *T2Hh*^{t} and *T2Hh*^{c}. *T2Hh*^{t} produces very faint plaques on *R/2H*, and only about 20 percent as many on this bacterium as on the sensitive strains. *T2Hh*^{c} produces clear plaques to a count of 80 percent or so of the titre on sensitive bacteria. Both produce plaques indistinguishable from those of *T2H* on *S*. Both undergo *r* mutations, yielding *T2Hh*^{t}*r* and *T2Hh*^{c}*r*, which have about the same relative infectivity as the corresponding r^+ forms, but produce typical *r* plaques.

T2Hrh^t and *T2Hrh*^{c} can also be isolated from *T2Hr* by selecting the *h* mutants, and the resulting phages seem to be identical with those obtained through the reverse mutational sequence.

If the *h* and *r* mutations are truly independent of each other, the rates of the parallel mutations ought to be the same. The rates of *r* mutation cannot be measured conveniently, because we have no method for counting small numbers of *r* mutants in the presence of an excess of wild type. The rates of the *h* mutations can be measured, however, subject to certain technical difficulties.

The rate problem is essentially the same for phage as for bacteria, and our understanding of it is owing entirely to the work of LURIA and DELBRÜCK (1943) on bacterial mutation. A purely formal difference from their analysis is required because of our lack of knowledge of the mode of intracellular growth of phage (cf. LURIA 1945a). If we define the chance of a mutation occurring among N phage particles during the population increment dN by

$$adN, \tag{1}$$

so that a is the probability of mutation per phage particle per act of duplication, a is a suitable measure of mutation rate, and its experimental determination requires no assumption about the nature of growth or mutation. From (1) we get

$$a = \frac{-\log_e P_0}{N - N_0}, \tag{2}$$

which is equivalent to the equations (4) and (5) of LURIA and DELBRÜCK (1943). Similarly, from the differential equation for the growth of mutants

$$dh = a\,dN + \frac{h}{N}\,dN, \tag{3}$$

in which a dN is the contribution from the mutations and (h/N) dN is the contribution from the growth of progeny of previous mutations, to the total increment of mutants dh, we get

$$h = aN \log_e(NCa), \tag{4}$$

identical with their equation (8). If the mutant phage are enumerated with an efficiency E less than unity, one uses instead of (4)

$$h = aEN \log_e(NCa). \qquad (5)$$

These equations are subject only to the restrictions stated by LURIA and DELBRÜCK (1943). We now discuss their use in estimating rates of mutation of phage by two methods.

In the first method, a number of samples of phage, starting from amounts N_0 too small to contain any mutant progeny, are propagated in separate cultures of susceptible bacteria, the amounts and conditions of lysis being chosen so that only a fraction of the cultures will yield any mutants at all. After allowing the cultures to lyse, determining the average total number of phage particles N per lysate from a few tubes, and plating the entire contents of the remaining tubes with bacteria resistant to the parent phage, one determines the mutation rate a by equation (2), in which P_0 is the fraction of tubes yielding no mutants.

This method has the advantage that the relative growth rates of parent and mutant phage need not be known. It has the practical disadvantage that the method fails unless P_0 falls between, say, 0.3 and 0.9, which requires a careful choice of conditions of lysis. Its use is also restricted by the requirement that mutant phage be detectable with nearly perfect efficiency, for (2) is not a usable function of infectivities less than unity in the absence of information about the distribution of numbers of mutants per sample.

The second method differs only in the use of larger cultures of any convenient size, each of which is sampled for the enumeration of mutant phage particles, and a few for the titration of total phage. The mutation rate is given by equation (5), in which h is the average number of mutant phage particles (per culture) giving rise to plaques when C cultures each containing N total phage are plated with an efficiency E on bacteria resistant to the parent phage.

The second method is applicable only if the growth of parent and mutant phage proceeds at identical rates (or, by extension of the theory, if the relative rates are known) during lysis under the conditions of the experiment. One must pay attention especially to the possibility that bacterial mutants may arise in some tubes prior to lysis, on which the mutant phage may grow preferentially, even though there is no selection on the parent bacterial strain. This contingency can be avoided by proper attention to time of sampling. For the rest, it is necessary only in practice to show that in mixed lysates prepared by seeding bacterial cultures with known amounts of both parent and mutant phage, the ratio of the one to the other remains unchanged, except as influenced by further mutations. An example of the use of this method will be shown below.

The principal difficulty in applying either of these methods to the measurement of rates of h mutation of phage results from the fact, previously mentioned, that one gets different mutants with different infectivities for the indicator strain used for assay. With *T2H* there are at least two mutants, having infectivities of about 0.2 and 0.8, respectively. These can be differ-

entiated by the appearance of plaques (turbid and clear), but the variation in appearance of plaques of each type prevents a clean separation for counting. It will probably prove possible, by a suitable choice of conditions of assay, to make this separation. In the meantime, we have done preliminary experiments in which we count principally but not exclusively the h^c mutant which serve to answer the question: are *h* and *r* mutations of *T2H* independent of each other.

For this purpose we seed a flask of broth with sensitive bacteria, and with amounts of *T2H* too small to contain any h mutants. The seeded broth is dis-

TABLE 1

Proportion of h mutants arising in stocks of T2H and T2Hr.

PHAGE PER TUBE	6.9×10^9 *T2H*				4.5×10^9 *T2Hr*			
SET NO.	1	2	3	4	1	2	3	4
Tube No.	Clear plaques on *R/2H,2K*				Clear plaques on *R/2H,2K*			
1	3	12	506	131	20	19	88	7
2	9	9	4	3	21	10	35	5
3	3	2	139	227	6	134	5	60
4	10	22	99	4	155	38	16	37
5	5	39	1	260	78	7	41	0
6	189	8	110	31	33	94	1	23
7	8	1	38	68	24	42	18	1
8	0	53	36	516	3	14	1	0
9	23	226	39	113	9	16	0	43
10	237	1	79	14	70	34	86	604
Mean (h)	48.7	37.3	105	137	41.9	40.9	29.1	78.0
Variance (σ^2)	6,910	4,240	19,700	28,400	1,990	1,540	1,020	31,100
σ^2/h, exp.	142	113	187	206	47	38	35	399
σ^2/h, calc.	22.3	18.7	37.8	45.0	20.8	20.0	16.1	30.6
Mutation rate$\times10^9$	1.5	1.2	2.9	3.6	2.1	2.0	1.5	3.4

Each tube contained initially about 10^4 bacteria and about 10^4 phage, in a volume of 0.5 cc After 24 hours, two tubes of each kind were assayed for total phage, and the entire contents of the remaining tubes were plated with resistant bacteria. Only clear plaques were counted (the turbid ones being scarcely visible) and the counts were recorded in random sets of ten. The ratio σ^2/h would be unity if the variations in counts were due to sampling errors alone. The calculated value of this ratio, on the hypothesis of prior mutation, was obtained by the equation (12a) of LURIA and DELBRÜCK (1943).

tributed in tubes to contain 0.5 ml each, and the cultures are then allowed to lyse. Twenty-four hours later the total phage is assayed in a few tubes by plating samples of suitable size on the sensitive bacterium. These counts do not vary appreciably from tube to tube. The entire contents of the remaining tubes are assayed for *h* mutants by plating with the indicator strain *R/2H, 2K* (preferable for this purpose to *R/2H*). To do this one adds to each tube 3.5 ml of 0.6 percent agar containing the bacteria, and pours the mixed contents onto the surface of solidified agar (HERSHEY, KALMANSON, and BRONFENBRENNER

1944). Similar experiments, starting with *T2Hr* instead of wild type, are done in the same way. Further quantitative details, and the results of the assays, are shown in table 1.

The results show that the parallel mutations *T2H*→*T2Hh*^{c}, and *T2Hr* →*T2Hrh*^{c}, occur with the same frequency, and therefore that the structural changes in the phage particle corresponding to the *h* and *r* mutational effects occur independently of each other.

It remains only to check whether *T2H* and *T2Hh*^{c} compete on an equal basis during growth under the conditions used above. This can be done for both lysis-inhibiting and *r* stocks as follows. Cultures of the sensitive bacterium in broth are seeded with mixtures of host range mutant and corresponding wild type in the proportion of about 1:10^5. This proportion is sufficiently small to approximate the conditions of the rate measurements, but too large to be appreciably affected by further *h* mutations. The mixture of phages used for seeding is plated on the sensitive bacterium, and on *R/2H*, to enumerate the

TABLE 2

Competitive growth of h$^+$ *and h mutant on E. coli.*

	PLAQUES ON *S*	PLAQUES ON *R/2H*	RATIO h^c/h^+
	Mixture of *T2H* and *T2H*^{c}		
Inoculum	4.8×10^5	4.4×10^0	9.2×10^{-6}
Lysate	3.4×10^{10}	2.8×10^5	8.2×10^{-6}
	Mixture of *T2Hr* and *T2Hrh*^{c}		
Inoculum	8.8×10^5	2.3×10^0	2.6×10^{-6}
Lysate	1.3×10^{10}	2.8×10^4	2.2×10^{-6}

Plaque counts are expressed per ml of the culture tube.

two component types. After lysis of the seeded cultures, counts are made in the same way of the mixed progeny. The results (table 2) show that no significant change occurs in the proportion of *h* mutant. The two types of phage therefore grow without selective interaction, and the data of table 1 can be used for estimating rates of h mutation.

By equation (5) these rates are, for the mutation *T2H*→*T2Hh*^{c}, 1.2 to 3.6×10^{-9} in wild type, and 1.5 to 3.4×10^{-9} in *r* mutant, calculated for individual sets of ten cultures, assuming E = 1.0. The counts of mutants in individual cultures showed the large fluctuations to be expected for spontaneous mutations in agreement with the experience of LURIA (1945a) with *h* mutations in *T1* and *T2L*.

GROWTH CHARACTERISTICS

One-step growth experiments (DELBRÜCK and LURIA 1942) were made with *T2H* and its *r* mutant. The phages were added to a twofold or greater excess of bacteria, so that the cells were infected with single particles of phage. In one experiment, the simultaneous growth of both phages was followed in a single

tube, counting separately the two kinds of plaque. The experiments, which agreed well, are summarized in table 3. There was no difference in the growth of the two phages except for the delayed lysis in the adsorption tube with *T2H*, and a somewhat larger burst-size with this phage as compared with the *r* mutant. Both phages showed a rather indefinite rise period. The bulk of the phage was liberated during the first ten minutes after the end of the latent period, but this was followed by a gradual rise lasting 20 minutes or more. This indicates sluggish lysis of some of the bacteria, or progressive disintegration of bacterial fragments. Evidently the *r* mutation, which alters the rate of lysis of reinfected bacteria in the adsorption tube, does not affect the lysis of singly infected ones in diluted culture.

TABLE 3

Growth of T2H and T2Hr on singly infected E. coli strain H.

PHAGE	NO. OF EXPERIMENTS	ADSORPTION IN 5 MINUTES	LATENT PERIOD	RISE PERIOD	BURST SIZE	LYSIS-TIME IN ADSORPTION TUBE
		percent	minutes	minutes		hours
T2H	2	61	21	30	420	5
T2Hr	3	66	21	30	330	1

COMPETITIVE GROWTH AND BACK MUTATION

In contrast with the behavior of the *h* mutants previously described, the *r* mutant shows a decided disadvantage in competitive growth with wild type. Experiments showing this were done with *T2H*. Two tubes containing 10 ml beef extract broth (0.1 percent glucose) were seeded with susceptible bacteria (*H*) and allowed to grow to a population of about 10^7 bacteria per ml. At this point mixtures of the two phages, containing respectively 2 percent *T2Hr* in *T2H* (series A), and one percent *T2H* in *T2Hr* (series B), were added. After lysis was complete, and usually after standing overnight in the incubator, the lysates were freed from any resistant bacteria they might contain by heating at 60°C for ten minutes, samples were plated, and new cultures were inoculated from them to yield a second pair of lysates, and so on. The tubes were seeded each time with about 0.02 ml of the preceding lysate. The two types of phage in mixtures could be counted with fair accuracy by pouring plates with two aliquots yielding about 100 and 500 plaques, respectively. The changes in the composition of the population during several serial transfers with each mixture are shown in table 4. With wild type in excess (series A), the proportion of *r* mutant decreases slowly to the level characteristic of stocks of wild type, near one per thousand. With *T2Hr* in excess (series B), the mutant is rapidly replaced by wild type. The increase in the proportion of wild type under these conditions is twofold or greater for each five-hundredfold increase in phage. This is clearly in excess of the difference in burst-size observed earlier, which could account only for a 30 percent increase.

The competitive advantage of wild type over *r* mutant provides a method for detecting the back mutation. To this end we have made successive serial lysates starting from a single plaque of a pure stock of *T2Hr* (table 4, series C), in parallel with a series propagated under identical conditions starting from a mixture containing only three wild type per 10^8 phage particles (series D). In the latter series, lysis inhibition was evident at the eleventh transfer, and in series C at the twelfth transfer. In both cases, the presence of phage resembling wild type was confirmed by plating and isolating the phage. The line obtained

TABLE 4

Competitive growth of T2H and T2Hr on E. coli strain H.

SERIES	NO. OF SERIAL TRANSFER	LYSIS INHIBITION	PHAGE TITER	PROPORTION OF r^+ PLAQUES
A	inoculum		1.0×10^5	0.98
	1	+	9.3×10^9	0.99
	2	+	4.4×10^{10}	0.99
	4	+	2.7×10^{10}	0.995
	5	+	4.6×10^{10}	0.998
B	inoculum		1.0×10^5	0.01
	1	−	4.6×10^{10}	0.03
	2	−	1.5×10^{10}	0.08
	3	+	2.5×10^{10}	0.1
	4	+	2.6×10^{10}	0.2
	5	+	1.8×10^{10}	0.5
	8	+	1.6×10^{10}	0.96
C	inoculum		fished from plaque	0.0
	1 to 11	−	not plated	
	12	+	6.5×10^9	0.1
D	inoculum		3.0×10^7	3×10^{-8}
	1 to 10	−	not plated	
	11	+	1.4×10^{10}	0.2

in this way proved, on analysis of host range, to be authentic *T2H*. Its identity with wild type was further established by a comparison of rates of mutation to *r* (table 5). Plaques of the back mutant, and of the *r* mutant rederived from it, were indistinguishable from those of the corresponding derivatives of wild type. Back mutation of a similar kind has been observed in every instance tried, including some with genetically marked phages, and the possibility of contamination can be excluded.

The amount of phage transferred from tube to tube in series C and D (table 4) was 0.05 ml, corresponding to about 5×10^8 phage particles. By decreasing this amount until successive transfer would fail to bring out wild type, the rate of back mutation could be estimated roughly—hence this rate must exceed one in 5×10^8. On the other hand, series D reverted faster than series C—hence,

the rate is less than one in 3×10^7. The rate of back mutation to wild type is therefore of the order 10^{-7} or 10^{-8}, as compared with about 10^{-3} for the mutation from wild type to *r* (table 5).

An attempt was made to learn something about the competitive interaction of wild type and *r* mutant in individual bacteria by means of experiments similar to those devised by DELBRÜCK (1945a) to demonstrate the mutual ex-

TABLE 5

Proportion of r mutant in lysates of T2H and back-mutant from T2Hr.

LYSATES	TITER	*r* PLAQUES / TOTAL INSPECTED
	$\times 10^{10}$	
wild type		
1	1.1	2/640
2	0.89	1/530
3	1.2	0/710
4	1.1	0/640
5	1.4	0/850
Mean	1.1	1/1120
back mutant		
1	1.2	0/730
2	0.96	2/580
3	1.0	0/620
4	0.89	1/530
5	0.63	1/380
Mean	0.95	1/710

Each lysate represents the progeny stemming from a different plaque of the respective phages.

clusion effect. Bacteria (strain H) were allowed to grow in broth to a population of about 3×10^7 per ml at which time a mixture containing an excess of the two phages was added. After five minutes for adsorption of phage, one sample of the culture was diluted in 0.1 percent peptone water and spun for the assay of unadsorbed phage, and a second sample was diluted 1:50 into 1:100 antiserum (anti-*T2K*). After five minutes for reaction with antibody, during which virtually all the unadsorbed phage was neutralized (DELBRÜCK 1945b), this sample was diluted again by the factor 2500 into broth, and samples were plated with sensitive bacteria both before (aliquots 2 and 5×10^{-6} ml with respect to the adsorption mixture) and after (aliquots 50 times smaller) lysis of the infected bacteria. For comparison, a similar experiment was done with bacteria multiply infected with *r* mutant alone. The results of the three experiments (table 6) were consistent in all important respects; all the bacteria were infected with several particles of phage, and the count of bacteria by colonies or

by infective centers before lysis agreed very well, showing that the free phage had been neutralized. A striking result of mixed infection was the appearance of a new type of plaque, of large size like the *r* plaques, but heavily mottled in characteristic fashion with patches of unlysed bacteria (fig. 1). These proved invariably to contain approximately equal numbers of wild type and *r* phage. Fishings from the typical appearing r^+ or *r* plaques, on the other hand, contained nearly pure wild type, or pure *r* mutant, respectively.

TABLE 6

Mixed infection with T2H and T2Hr (experiments 1 and 2), and multiple infection with T2Hr alone (experiment 3).

DATUM	MINUTES	EXPT. 1 COUNT OF PLAQUES OR COLONIES				EXPT. 2 COUNT OF PLAQUES OR COLONIES				EXPT. 3 COUNT OF PLAQUES OR COLONIES (*r* ONLY)
		r^+	*r*	MIXED	TOTAL	r^+	*r*	MIXED	TOTAL	
Bacteria	−5				57				75	97
Phage added	0	200	360			423	344			1100
Phage adsorbed	5	110	230			312	222			980
Adsorbed per B		1.9	4.0			4.2	3.0			10
Infective centers	<15	3.8	15	40	59	20	15	50	85	91
Phage yield	60	374	620			2780	2480			5800
Yield per B		6.3	11		17	33	29		62	64
r^+/r input		0.56				1.2				
r^+/r adsorbed		0.48				1.4				
r^+/r yield		0.57				1.1				

All counts are expressed per 2×10^{-6} ml of adsorption tube, from the mean of three or four plates each for two different aliquots.

In all three experiments, the yield of phage after one step growth was low, as compared with the yield in single infection. This is probably due to the fact that dilutions were made at room temperature, in 0.1 percent peptone water, and to the poor nutrient conditions during the reaction with antiserum, which was carried out at 37°C in the same fluid. Three additional experiments were done in parallel specifically to compare the yield following multiple infection with wild type alone, *r* mutant alone, and a mixture of the two, under standard physiological conditions. The schedule of the previous experiments was followed precisely, except that certain nonessential measurements were omitted, and the dilutions and reaction with antiserum were all carried out at 37°C in broth. The yield of phage was indeed improved (table 7), though still only about half the yield in single infection. In all other respects the results of these experiments are in excellent agreement with those of table 6.

The conclusions to be drawn from the experiments with multiple infection may be summarized as follows.

As one varies the proportionate input of *r* mutant to wild type, the number of mixedly infected bacteria yielding wild type alone, or *r* mutant alone, varies

TABLE 7

Simultaneously Measured Yields of T2H and T2Hr in Multiple Infection.

INFECTING VIRUS	*T2Hr*	*T2Hr* AND *T2H*			*T2H*	
PLAQUE-TYPE	*r*	r^+	*r*	MIXED	r^+	*r*
Bacterial count	109					
Phage added*	700	350	350		700	(1)
Adsorbed per B*	4.8	2.4	2.4		4.8	
Infective centers at 12–20 Min.	127	17	25	75	(317)†	(1)
Yield at 60 Min.:						
Growth tube 1	16,300	9,800	10,300		16,600	(200)
Growth tube 2	15,200	8,300	9,100		18,800	(100)
Yield per B	130	75	80		147	(1)
r^+/r input		1.0				
r^+/r yield		0.94				

* Input adjusted from prior counts: 75 percent adsorption assumed.

† Sample plated at 21–22 minutes; evidently growth of phage had begun. See legend table 6.

in the expected direction. These numbers are about equal for 1:1 infection, indicating no superiority of the one over the other with respect to mutual exclusion (DELBRÜCK 1945a). This is evidently another expression of the near relatedness of the two phages.

The mutual exclusion effect itself is weak, for the majority of bacteria yield both types of phage, and evidently both grow. Another interpretation might be made in terms of directed mutation, under the influence of excluded virus, among the progeny of a single successful particle (DELBRÜCK 1946b). The quantitative data, particularly the identity of input and yield ratios following mixed infection, do not support this interpretation, but do not really decide the question.

The ratio of wild type to *r* mutant resulting from one-step growth in mixedly infected bacteria is identically equal to their ratio in the infecting mixture for the three ratios tested—namely, 0.6, 1.0, and 1.2. From the other data, and since the mixed yielders comprise 59 to 68 percent of the mixedly infected bacteria, the mixed yielders as a class must also conform to this rule.

The total yield of phage from bacteria multiply infected with wild type alone, *r* mutant alone, or a mixture of the two, is the same; about 150 particles per bacterium. There is, therefore, no depressor effect (DELBRÜCK 1945a), and the two phages grow as if they were competing on an equal basis for a common substrate, without other interaction.

The total yield of phage in multiple infection is about half that found in single infection, with either wild type or *r* mutant. However, the two sets of experiments were separated by an interval of about a year, and we have not determined whether the difference is an effect of multiple infection itself, of the antiserum, or of some unknown variable.*

The experiments on mixed infection under defined conditions do not reveal any mechanism adequate to explain the superiority of wild type over *r* mutant during competitive growth in mixed culture. The tendency for pure stocks of wild type to reach higher titers in broth, noticeable in tables 1 and 2 and consistently seen with other even-numbered phages also, is probably related to the selective effect in mixed culture, but it likewise is without explanation.

HOST SPECIFICITY

The question of the identity of host range of wild type and *r* mutant was investigated very thoroughly for all the even-numbered phages of the *T* system and for several *h* mutants of *T2H*, comprising eight r^+ stocks in all, and their corresponding *r* mutants. With each of these, ten or more independently arising phage-resistant bacterial mutants were isolated, by methods similar to those of DEMEREC and FANO (1945). As noted by these workers, mutants of *B* resistant to *T2* are difficult to obtain. These were isolated with little difficulty, however, by first growing sensitive bacteria in the presence of phage in salt-free broth for a few days (which selects partially resistant mutants), and then transferring phage and bacteria to broth containing 0.5 percent salt. After incubating these cultures for about two days, streak plates from them nearly always yielded stable resistant mutants.

The principal types obtained have already been mentioned in the section of this paper headed "materials." Other types included these same patterns in combinations of two or more linkage groups. Among these the most frequent were R/*2, 3, 4;* R/*2, 3, 4, 7, 16;* R/*2, 3, 4, 6, 7, 16;* and R/*2, 6, 16*. As in the other cases, resistance to *T2* nearly always included *T2H*, *T2K* and *T2L*.

Tests of sensitivity to phage were made by flooding the surface of agar plates with broth cultures of the bacteria to be tested, drying the plates by inverting them in the incubator with covers removed for one hour, and then placing small drops of suitably diluted phage from capillary pipettes on the surface of the seeded agar. The drops were evaporated down by again exposing the plates for 20 minutes in the incubator. In many cases several dilutions of the phages were tested. A culture was arbitrarily classified as resistant when a drop of phage containing about 10^6 phage particles produced no marked confluent lysis and yielded fewer than 500 plaques. Usually there was no effect whatever at this dilution on resistant cultures, and more or less complete lysis with sensitive ones.

* Subsequent experiments have shown that certain lots of nutrient broth give low yields of virus, the maximum for *T2H* being about 400 particles per bacterium in single infection. Using a given lot of broth, the yield is reproducible, is not affected by antiserum, and is larger rather than smaller following multiple as compared with single infection.

None of the 200 or more cultures tested, including some dysentery strains, and some derivatives of strains of *E. coli* unrelated to *R*, distinguished sharply between any wild type phage and its *r* mutant. With some incompletely resistant strains, there were quantitative differences in infectivity of the two types. This kind of difference is of doubtful significance for the reason that similar differences are seen between different stocks of the same type, depending for one thing on the proportion of *h* mutants they contain.

One extreme case of this kind was examined in detail. The bacterial strains *R/2K, 6, 16* and *R/2H, 2K*, resistant to *T2K*, proved to be sensitive to our stock of *T2Kr*. But when new stocks of each type were prepared, starting from a single variegated plaque of wild type, the difference disappeared. We conclude that our stock of putative *r* mutant had also undergone an *h* mutation, either before or after its origin from the r^+ type. The results of tests with these stocks are shown in table *8*, which will serve to illustrate the method we have used for analysis of host specificity. Tests with *T2H* and *T2L* are included for comparison.

TABLE 8

Spot tests on seeded agar with T2K and T2Kr.

BACTERIAL STRAIN		*R*	*R*	*R*	*R*	*D*
PHAGE	TITER PER DROP	*2H, 2L*	*2H*	*2H, 2K*	*2K, 6, 16*	*2K, 3, 4, 7*
T2K	3×10^6	C	C	—	2	—
T2Kr1	3×10^6	C	C	C	C	1
T2Kr2	3×10^6	C	C	—	1	—
T2K	3×10^4	>1000	100	—	—	—
T2Kr1	3×10^4	>1000	1000	200	>1000	—
T2Kr2	3×10^4	>1000	50	—	—	—
T2K	3×10^2	30	—	—	—	—
T2Kr1	3×10^2	100	20	2	100	—
T2Kr2	3×10^2	20	—	—	—	—
T2H, r^+ or *r*	3×10^5	—	—	—	C	C
T2L, r^+ or *r*	3×10^5	—	C	C	C	C

T2Kr1 was isolated in 1944; *T2Kr2* is an independent mutation recently isolated from *T2K*. *D/2K, 3, 4, 7* is a wild type *Shigella paradysenteriae*.

The symbol C indicates complete lysis, numbers refer to plaques, and — indicates no effect.

Since the tests described above were completed, we have had occasion to examine a rather large number of independently arising *r* mutants of several phages. These have never differed in host range from the parent stock, which makes it unlikely that coupled *h* and *r* mutations ever occur.

In general, the analysis of host specificity of r^+ and *r* phages has merely confirmed our earlier conclusion that the *h* and *r* genetic factors of bacteriophage are mutually independent.

ANTIGENIC SPECIFICITY

Neutralization tests with several antisera failed to show any antigenic difference between wild type and *r* mutant. Absorption tests were therefore resorted to, the results of which will be given here only in condensed form.

The methods of serological analysis used in these experiments have been described in detail elsewhere (HERSHEY, KALMANSON, and BRONFENBRENNER 1943). They are based on the measurement of the specific rate of neutralization (k) per second, which for the present purpose may be defined as a number proportional to $(1/2240\ \times)$ the dilution of serum which will neutralize 80 percent of added phage in one hour under specified conditions. This measurement can easily be duplicated with an accuracy of 20 percent. With homologous phage and antiserum, k provides a measure of the antibody content of the serum. In heterologous reactions, the measured k is a plausible index of antigenic relatedness.

It was shown by BURNET (1933b) that absorption with heterologous phage increases the specificity of the antiserum, as is the case with other antigens. He used phage adsorbed to killed bacteria for the absorbing antigen. His method is of limited applicability for the reason that many phages, *T1* for instance, are not adsorbed to dead bacteria. We have made adsorptions with phage alone, which is practicable at least with *T1* and the even-number phages of the *T* system.

For the even-numbered phages, the appropriate conditions for absorbing 50 to 80 percent of the antibody from antisera at 37°C in broth containing 0.5 percent NaCl are met when the ratio of antibody to phage is

$$\frac{\text{ml antiserum} \times \text{k}}{\text{lytic units of phage}} = 2 \times 10^{-11}$$

The mixture should contain about 10^{10} lytic units per ml, in which case precipitation occurs in a day or two, and the sediment can be removed before titration. With more dilute mixtures, no visible precipitate forms, but the results of titration are very similar. At low temperatures the reaction is slower, but considerably more antibody is absorbed at equilibrium. The specified conditions are equally suitable for homologous or heterologous absorption, provided the appropriate k is substituted in the formula given above. Mixtures prepared under these conditions contain only negligible amounts of active phage, but if it is desired to avoid this, one can carry out the absorption with phage previously inactivated with minimal amounts of formaldehyde.

The results of tests made with several phages are summarized in table 9. The data are expressed in terms of the ratio of the specified heterologous k to the homologous. For example, *T4* in whole anti-*T2K* yields k = 1.0, and *T2K* in the same serum yields k = 4.5, the ratio 0.22 being entered in the table. This ratio expresses the relatedness of the phages on the one hand, and on the other measures a quality of the antibody, a lower ratio indicating greater specificity. It may therefore be taken as a criterion of antigenic difference between phages,

that heterologous absorption will lower this ratio of k's. This effect is more significant than the absolute value of the ratio, which might conceivably be influenced by causes other than antigenic dissimilarity (no case of this kind has been encountered, however).

Similar considerations apply to the reliability of measurements. For instance, the ratio 1.3 recorded in table 9 for the neutralization of *T2H* by anti-*T2K* is certainly not significantly different from unity, since it is the consequence of a single aberrent result (1.6) among three titrations and is in any case a deviation in the opposite direction to that found with heterologous phages. Accordingly, one finds no significant effect on this ratio of absorption with *T2H* or *T2L*, all three titrations being influenced by the same error. This is an expres-

TABLE 9

Antigenic relationships among even-numbered and mutant phages.

SERUM-FRACTION TESTED	RATIO OF HETEROLOGOUS TO HOMOLOGOUS TITER AGAINST					
	T2H	*T2Hrh*^{c}	*T2L*	*T2Kr*	*T4*	*T6*
Anti-*T2K*, whole	1.3_3	0.97_1	0.72_5	0.96_7	0.22_5	0.20_2
Anti-*T2K*/*T2H*	1.3_2					
Anti *T2K*/*T2L*	1.3_1		0.60_5		0.40_6	
Anti *T2K*/*T2K*				0.95_5	0.67_2	
Anti-*T2K*/*T2Kr*				0.93_7	0.69_2	
Anti-*T2K*/*T4*	1.0_1		0.87_6	1.1_2	0.04_8	0.03_8
Anti-*T2K*/*T2Hrh*^{c}		1.2_4				
Anti-*T2Hr*, whole	1.03_2					
Anti-*T2Hr*/*T2H*	0.94_2					

Anti-*T2K*/*T2H* means the serum of a rabbit immunized with *T2K* from which 50 to 90 per cent of the antibody has been absorbed with *T2H*, etc.

The subscript numerals indicate the number of independent measurements of the specified ratio contributing to the average result shown.

sion of the technically important fact that simultaneous titrations of two antisera with the same phage are subject to less relative error than simultaneous titrations of two phages in one antiserum, or successive titrations of the same materials (HERSHEY, KALMANSON, and BRONFENBRENNER 1943). For this reason, we arrange our titrations to include in each run the sera it is desired to compare, against the homologous and one or two heterologous phages.

From the same considerations (and since a similar relationship was evident in each of the individual titrations), the very small effects of heterologous absorption on the neutralization of *T2L* can be taken as evidence of a slight antigenic difference between this phage and *T2K*.

Summarizing the data of table 9 we find:

T2K and *T2H* are indistinguishable in anti-*T2K* antiserum. *T2L* is slightly, and *T4* and *T6* are very much, different from these. *T16* (data not shown) is still further removed, judging by rate of neutralization alone.

T2Hr and *T2Kr* are indistinguishable from the corresponding wild types in antiserum against either mutant or wild type.

An *r* mutation followed by *h* (*T2Hrh*^{c}) does not result in any cumulative antigenic difference. This conforms with our failure to distinguish *h* mutant from wild type *T1* by absorption test (data not shown).

Absorption of anti-*T2K* with *T4* removes selectively antibody which reacts with *T4* and *T6*, but not (or less rapidly) with *T2L*. This implies a component antigenic pattern common to *T2K*, *T4*, and *T6*, which is different in *T2L*.

Absorption of anti-*T2K* with *T2L* removes selectively antibody which reacts with *T2L*, but not with *T4*. This implies an antigenic component common to *T2K* and *T2L*, which is different in *T4*.

Absorption of anti-*T2K* with *T2K* (or *T2Kr*) leaves behind a fraction of antibody that reacts almost equally well with *T4* and *T2K*. This confirms in a remarkable way some of the conclusions stated above.

T4 and *T6* are indistinguishable in anti-*T2K* antiserum, although they are easily distinguishable in their own. Evidently these two phages have the same antigenic component in common with *T2K*. This component is probably not associated with the *r* locus, for *T4* and *T4r* cannot be distinguished in anti-*T2K* after absorption with either one (data not shown). The relationship of *T16* with respect to this component could not be conveniently determined, because of the very low titer of anti-*T2K* against this phage.

DISCUSSION AND SUMMARY

Phages belonging to the *T2* (even-numbered) series, form a group that is morphologically homogeneous and whose members are antigenically related; they are dependent for their infectivity on relatively high concentrations of univalent cations, which in general inhibit other members of the *T* system (unpublished experiments), and they are characteristically sensitive to urea, but are comparatively resistant to heat and certain other agencies (BURNET 1933c).

In addition, these phages as encountered in nature exhibit a unique lysis-inhibiting property recognizable by delayed lysis in broth and only partial lysis in the peripheral zone of high phage concentration surrounding plaques in agar. From the wild types (r^+) one regularly obtains mutants (*r*) which fail to show either of these lysis-inhibiting effects, or show them to much smaller degree. The frequency of the *r* mutation is about 10^{-3} or 10^{-4}.

The *r* mutant, in turn, mutates back to wild type, but with much lower frequency, probably about once per 10^8 duplications. The important question, whether this is a true back mutation—that is, whether the recovered lysis-inhibitor is identical with the original wild type—seems to be answered in the affirmative by the finding that both mutate to *r* with similar frequency. This latter evidence is reasonably decisive and leads to the conclusion that *r* mutation corresponds to a true allelic modification of structure rather than to loss of any genetic material.

When mixtures of wild type and *r* mutant are propagated in broth on bacterial strains equally sensitive to the two types by all other tests, wild type replaces *r*. Although the mechanism of this replacement is unknown, it is

evident that it explains the persistence of wild type in spite of the unfavorable balance of mutation rates. It also permits the demonstration of the back mutation, which would otherwise not be observable.

A mutation resulting in altered host range ($h^+ \rightarrow h$) has been found to occur at the same rate in r and r^+ stocks of one of these phages (*T2H*). This may be taken as proof of the existence of at least two independent genetic factors, and it is permissible to refer to these as h and r loci, provided the method of identification is kept in mind.

The existence of independent genetic factors does not alter the status of the analogy commonly drawn between viruses and genes. The mutational properties of the phage are precisely analogous to those of the *R* determinants of seed and plant color in maize, which always segregate together, but undergo mutation independently (STADLER and FOGEL 1943). In either case one has freedom of choice in speaking of multiple genes (that is, mutational units), or of independent functions of a single gene (the crossover unit). Evidently, with viruses as with bacteria (DEMEREC and FANO 1945), the only genetic factors amenable to study by present methods are the mutational units. For the present, then, no useful distinction can be made between the genetic behavior of a bacterium, a virus, and a crossover unit bearing two or more independently mutating genetic factors.

The antigenic specificity and the host specificity of wild type and r mutant are the same, and h mutation alone, or successive to r, also fails to alter serologic character. Evidently these three hereditary characters of the virus are independent of each other, or nearly so. The independence of h mutation and antigenic specificity is in keeping with what might be surmised from numerous instances of adaptation to unnatural hosts among the animal viruses, where a similar principle forms the basis of the classical methods of antiviral prophylaxis.

In connection with the serological experiments, an essay was made toward antigenic analysis of relationships among natural phage types by means of cross absorption tests. The results show that the methods available would permit the serological structure of bacterial viruses to be dissected in great detail. The conclusions from the preliminary experiments have been summarized in the experimental part of this paper and will not be repeated here.

Experiments in which bacteria are simultaneously infected with wild type and r mutant show the unexpected result that many bacteria yield both types of phage. For instance, if each bacterium receives about three particles of each type of phage, the population splits into about 20 percent wild type yielders, 20 percent r yielders, and 60 percent mixed yielders. The mixed yielders produce wild type and r mutant in the same ratio with which the bacteria were infected and in an amount which suggests that the two types are initiating growth and competing with equal efficiency for the same substrate. The proper interpretation of these results is not yet clear, however.

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